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Inducible Expression of Human Hepatitis B Virus (HBV) in Stably Transfected Hepatoblastoma Cells: a Novel System for Screening Potential Inhibitors of HBV Replication

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We report the development and isolation of a cell line, termed HepAD38, that replicates human hepatitis B virus (HBV) under conditions that can be regulated with tetracycline. In the presence of the antibiotic, this cell line is free of virus due to the repression of pregenomic (pg) RNA synthesis. Upon removal of tetracycline from the culture medium, the cells express viral pg RNA, accumulate subviral particles in the cytoplasm that contain DNA intermediates characteristic of viral replication, and secrete virus-like particles into the supernatant. Since the HepAD38 cell line can produce high levels of HBV DNA, it should be useful for analyses of the viral replication cycle that depend upon viral DNA synthesis in a synchronized fashion. In addition, this cell line has been formatted into a high-throughput, cell-based assay that permits the large-scale screening of diverse compound libraries for new classes of inhibitors of HBV replication.

Human hepatitis B virus (HBV) is a member of the *Hepadnaviridae* family, which is characterized by a circular, partially double-stranded DNA genome approximately 3,000 bp long, an enveloped capsid, and the ability to infect liver cells (10). After primary infection of the host, the virus may establish a chronic infection in the liver, which can, in turn, lead to cirrhosis and hepatocellular carcinoma (2, 21). It is estimated that of the approximately 300 million individuals worldwide who are chronic carriers of HBV, 1 million die annually from HBV-induced disease (18).

Currently, the only available treatment for chronic hepatitis in the United States is alpha interferon. Its efficacy, however, is partial and of limited duration. In clinical studies, approximately 30% of chronic carriers treated with interferon responded to treatment but the virus reappeared in greater than 50% of these patients within 2 to 3 months after cessation of treatment (8, 32). As an alternative approach, several nucleoside analogs have been investigated as potential inhibitors of HBV replication (reviewed in references 9 and 20). In early clinical trials, both lamivudine and ganciclovir have proven to be effective in decreasing the levels of HBV DNA in the serum of chronically infected patients (6, 11, 13). However, most patients relapsed shortly after therapy was discontinued, suggesting that the virus persisted in the liver during the entire trial period. As with human immunodeficiency virus, successful HBV therapy may depend on the simultaneous use of multiple compounds with antiviral activity.

The discovery of additional classes of inhibitors of HBV replication would be facilitated by the ability to screen large chemical libraries for compounds that interfere with specific steps of the viral DNA replication cycle. In the past, such efforts have been hampered by the lack of a suitable cell-based assay although several HBV-producing cell lines have been available for almost a decade (25, 27, 29–31, 33, 34). For example, the 2.2.15 cell line, which has been used to assay compounds for anti-HBV activity, constitutively produces HBV Dane particles, with maximum production occurring after the cells have become confluent (1, 25, 26). Since the subviral particles in the cytoplasm of these cells are relatively stable, cells must be exposed to the test compound for approximately 7 days before an antiviral effect can be detected. Moreover, because of the low level of virus replication in this cell line, the assay usually requires the use of a 24-well plate and thus depends on relatively large amounts of test compounds (15, 16). In an effort to circumvent these problems, we have produced the cell line HepAD38, which expresses HBV under the control of the inducible tetracycline promoter and shown that this cell line permits the rapid screening of entire compound libraries for inhibitors of the HBV DNA replication cycle in a 96-well format.

MATERIALS AND METHODS

Cell lines and culture conditions. HepG2 cells (14) and HepAD38 cells were maintained in Dulbecco's modified Eagle's/F-12 medium (DMEM/F-12; GIBCO BRL/Life Technologies, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (FBS), 50-μg/ml penicillin, 50-μg/ml streptomycin, 100-μg/ml kanamycin (PSK) at 37°C in 5% carbon dioxide. In addition, HepAD38 cells were grown in the presence of 0.3-μg/ml tetracycline and 400-μg/ml G418 (GIBCO BRL/Life Technologies). 2.2.15 cells (1, 24, 25), a kind gift from George Acs, were maintained in RPMI 1640 medium with 10% FBS, PSK, and 400-μg/ml G418.

HepG2 cells (4×10^5/35-mm-diameter plate) were cotransfected with 0.3 μg of pUHD15-1neo (12) and 2.7 μg of ptetHBV (Fig. 1) by using a liposome transfection kit as directed by the manufacturer (Transfectace; GIBCO BRL/Life Technologies). Transfected cells were selected in DMEM/F-12 medium containing 10% FBS, 1 μg of tetracycline per ml, and 400 μg of G418 per ml until individual clones could be identified by visual inspection of the plates (approximately 1 month). Individual clones were isolated and maintained in DMEM/F-12 medium supplemented with 10% FBS, PSK, 0.3-μg/ml tetracycline, and 400-μg/ml G418.

Plasmid constructs. Plasmid ptetHBV was created by removing the cytomegalovirus immediate-early (CMV-IE) promoter from pCMVhbv (7) and replacing it with the tetracycline-responsive CMV-IE promoter (CMVtet) from pUHD10-3 (12; Fig. 1). pCMVhbv was created by fusion of the sequence corresponding to the cDNA of pregenomic (pg) RNA of HBV, subtype *ayw* (3), to the CMV-IE promoter (4, 7). The nucleotide sequence of ptetHBV is available upon request.

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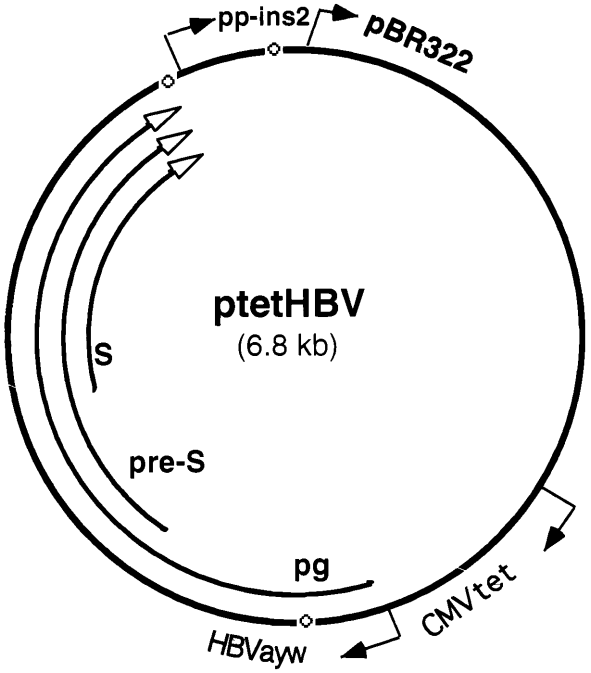

FIG. 1. Physical map of ptetHBV. Shown is a circular map of ptetHBV with the positions of the CMVtet promoter and HBV sequences. The segment marked pp-ins-2 is derived from preproinsulin gene 2, which contains a polyadenylation signal (4). The positions of the three major viral transcripts, pg RNA (pg), presurface mRNA (pre-S), and surface mRNA (S), are indicated by arrows. o, polyadenylation signal.

Isolation and analysis of polyadenylated RNA. Polyadenylated RNA was isolated from HepAD38 cells with a modified fast-track method (In Vitrogen, San Diego, Calif.). Briefly, cells were washed twice with phosphate-buffered saline (PBS) and lysed with 10 ml of lysis buffer (0.2 M Tris HCl [pH 7.5], 0.2 M NaCl, 2% sodium dodecyl sulfate, 25 mM EDTA, 0.2-mg/ml proteinase K) per T162 flask. The cell lysate was sheared by repeated (10 times) passage through an 18-gauge needle and incubated at 50°C for 1 h. Following addition of NaCl to the lysate to a final concentration of 0.5 M, the lysate was incubated with 60 mg of oligo(dT) cellulose (Stratagene, La Jolla, Calif.) in 1 ml of binding buffer (0.5 M NaCl, 10 mM Tris HCl [pH 7.5], 0.1 mM EDTA) on a shaking platform for 1 h at room temperature. The oligo(dT) cellulose was pelleted and washed four times with 10 ml of binding buffer, resuspended in 0.5 ml of binding buffer, and transferred to a spin column. RNA were eluted with 0.4 ml of elution buffer (10 mM Tris HCl [pH 7.5], 1 mM EDTA) at 65°C, precipitated with ethanol, and resuspended in 0.05 ml of diethylpyrocarbonate-treated water. One microgram of poly(A)-selected RNA was electrophoresed through a 1% formaldehyde agarose gel, transferred to a nylon membrane (Hybond-N; Amersham, Arlington Heights, Ill.), and hybridized with a ^{32}P-labeled HBV RNA probe.

Isolation and characterization of viral and chromosomal DNAs. To isolate viral DNA, we used a method described by Summers et al. (28). Briefly, cells from a 60-mm-diameter plate were lysed in 1 ml of lysis buffer (50 mM Tris HCl [pH 8.0], 10 mM EDTA, 150 mM NaCl, 1% sodium dodecyl sulfate, 0.5 mg of pronase per ml) at 37°C for 60 min, phenol extracted, and ethanol precipitated. The pellets containing nucleic acids were resuspended in 80 μl of TE (10 mM Tris HCl [pH 8.0], 1 mM EDTA). One quarter of each sample was electrophoresed through a 1.5% agarose gel, transferred to a nylon membrane, and hybridized with a radioactive RNA probe.

For the isolation of covalently closed circular (ccc) DNA, the cells were lysed in 1 ml of lysis buffer without pronase. To precipitate protein-detergent complexes, such as the DNA intermediates of HBV replication, 0.25 ml of 2.5 M KCl was added to the lysate. Following removal of the precipitate by centrifugation, the supernatant was extracted with phenol and ethanol precipitated. The pellets were resuspended in 80 μl of TE and analyzed as described above.

For the isolation of DNA from secreted virus particles, the medium from HepAD38 and 2.2.15 cell monolayers (60-mm-diameter plates) was clarified of cellular debris by centrifugation (Sorvall RT-6000D centrifuge, 2,000 rpm for 10 min). Virus particles were precipitated from the cleared supernatants in 6% polyethylene glycol (PEG) 8000, and the viral DNA isolated as described above.

Chemicals. Human alpha interferon, tetracycline, and β-2′,3′-dideoxycytidine (ddC) were purchased from Sigma Chemical Co. (St. Louis, Mo.). (±)-2-Amino-1,9-dihydro-9-[(1α,3β,4α)-3-hydroxy-4-(hydroxymethyl)cyclopentyl]-6H-purine-6-one (2′-CDG) was received from Jack Secrist (Southern Research Institute, Birmingham, Ala.). 2′-Deoxy-3′-thiacytidine (3TC) and 2′,3′-dideoxy-5-fluoro-3′-thiacytidine (FTC) were received from Raymond Schinazi (Emory University, Atlanta, Ga.).

Antiviral and cytotoxicity assays. HepAD38 cells were plated into 96-well microtiter plates (6×10^4 cells/well) and grown for 3 days in the presence of 0.3 μg of tetracycline per ml. On day zero, the cells were washed several times with PBS and treated with tetracycline-free medium that contained either a test or a control compound. Each test compound was screened at two, three, or six concentrations in quadruplicate. On day 3, the medium was removed and replaced with fresh medium containing the compound. Twenty-four hours later, the medium was collected and clarified by centrifugation (Sorvall RT-6000D centrifuge, 1,000 rpm, 5 min). Ninety microliters of clarified medium was assayed for the presence of HBV DNA by dot blot analysis as described previously, except that 1% Nonidet P-40 was added to the denaturing solution (15, 16). Radioactivity was quantified with a Bio-Rad GS-363 PhosphorImager. The concentration of a compound that inhibited HBV replication by 50 or 90% (the EC_{50} or EC_{90}, respectively) was determined by linear regression.

To determine the cytotoxic effects of test compounds, the cell monolayer was washed with PBS and tested for cell viability by using a cell proliferation assay kit as directed by the manufacturer (CellTiter 96 Non-Radioactive Cell Proliferation Assay; Promega, Madison, Wis.). The concentration of a compound that inhibited cell viability by 50 or 90% (the TC_{50} or TC_{90}, respectively) was determined by linear regression.

RESULTS

Isolation of cell lines that produce HBV under the control of tetracycline. It has not been possible to screen large, diverse libraries for new classes of anti-HBV inhibitors, since cell lines that produced high levels of HBV in an inducible fashion were not available. To create such a cell line, we transfected a single copy of the cDNA of the pg RNA of HBV strain *ayw* under the control of the tetracycline-responsive promoter (12) into HepG2, Huh-7, 293, and HeLa cells as described in Materials and Methods (Fig. 1). Plasmid ptetHBV, which contained this construct, was cotransfected with plasmid pUHD15-1neo, which encodes the *trans*-acting transcriptional regulator of the tetracycline-responsive promoter and neomycin resistance. Since ptetHBV did not contain a selectable marker, this plasmid was used in a ninefold molar excess over pUHD15-1neo to increase the probability of isolating clones containing both plasmids. Resistant clones were selected in the presence of G418 at a concentration of 400 μg/ml. Approximately 1 month following the transfection, 209 colonies, 118 derived from HepG2, 37 from Huh-7, and 54 from 293 cells, were isolated and expanded to permit the detection of HBV replication.

To determine which clones could support the replication of HBV, confluent monolayers of cells were grown for 4 days in the presence and absence of tetracycline. The cell culture medium was clarified by centrifugation and assayed for the presence of HBV DNA by dot blot analysis as previously described (15). The HBV-producing clone yield was as follows: 2 (1.7%) of 118 HepG2 colonies, 1 (2.7%) of 37 Huh-7 colonies, and 1 (1.9%) of 54 293 colonies. Transfection of HeLa cells did not yield any HBV-producing clones. With the exception of one of the two clones obtained from HepG2 cells, all clones lost the ability to express HBV upon continued passage of the cells. The remaining clone, termed HepAD38, was expanded and examined for stable production of HBV in response to tetracycline.

Tetracycline-responsive replication of HBV in HepAD38 cells. To determine whether the HepAD38 cell line expressed the appropriate RNA and DNA intermediates that are characteristic of HBV replication, we analyzed viral mRNAs, as well as intracellular viral DNA forms. For the analysis of viral RNA, confluent HepAD38 cells were maintained with and without tetracycline in the culture medium for 5 days. Polyadenylated RNA was extracted and analyzed by Northern blot analysis (Fig. 2, RNA). The results showed that in the presence of tetracycline, the cells expressed two major HBV-specific

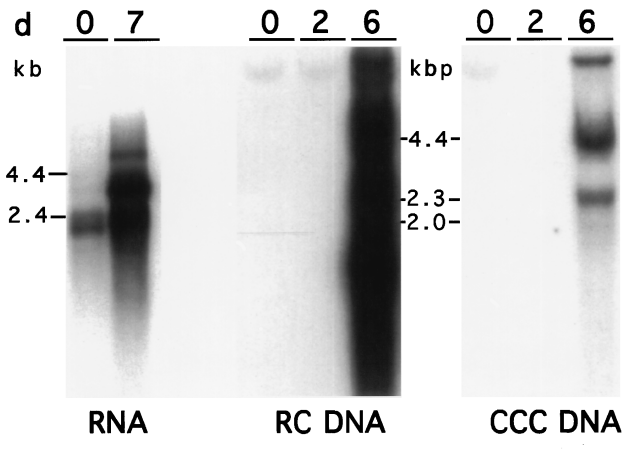

FIG. 2. Conditional expression of HBV-specific RNA and DNA forms in HepAD38 cells. HepAD38 cells were grown in the presence of tetracycline (1 μg/μl) until confluent. RNA and DNA were extracted from cells either 7 or 2 and 6 days (d), respectively, after removal of the antibiotic from the culture medium and analyzed by blot analysis. The positions of RNA and DNA size standards are indicated.

RNA species approximately 2 kb long, which corresponded to the presurface and surface mRNAs of 2.4 and 2.1 kb (Fig. 1). These mRNAs are expressed from two separate promoters on the viral genome and therefore are not expected to be regulated by tetracycline. In contrast, a major mRNA species approximately 3 kb long, corresponding to pg RNA, was expressed only when cells were grown in the absence of the antibiotic. Expression of minor mRNA species approximately 4 to 6 kb long was regulated by tetracycline like pg RNA. These may be derived from readthrough of the polyadenylation site on either the ptetHBV construct or the viral ccc DNA (Fig. 1). Thus, as expected from our experimental design, expression of pg RNA in HepAD38 cells occurred in a tetracycline-responsive fashion.

For the analysis of viral DNA, confluent HepAD38 cells were maintained in medium without tetracycline for 2 and 6 days prior to isolation of the nucleic acids. As expected from the results described above, viral DNA synthesis was completely inhibited in the presence tetracycline (Fig. 2). Upon removal of the antibiotic, the synthesis of viral DNA intermediates characteristic of HBV replication under natural conditions occurred with a latency period of at least 2 days. At day 6 following induction, DNA forms covalently linked to protein were present that corresponded to relaxed circular (rc) DNA, double-stranded linear (ln) DNA, and single-stranded (ss) DNA (Fig. 2, RC DNA). In addition, we detected DNA forms approximately 2.2 and 4.3 kbp long that were isolated by phenol extraction without prior incubation with pronase that may correspond to ccc DNA and nicked ccc DNA, respectively (Fig. 2, CCC DNA). We verified that the HBV DNA isolated from the nuclei of the induced HepAD38 cells was indeed ccc DNA by digestion of the DNA with *Dpn*I and exonuclease I (data not shown).

Comparison of viral DNAs expressed in HepAD38 and 2.2.15 cells. The use of the HepAD38 cell line in a high-throughput screen for inhibitors of HBV replication depends on obtaining levels of HBV production high enough for detection of HBV DNA in the medium of cells grown in the absence of tetracycline for only a few days in 96-well microtiter plates. To determine the level of HBV DNA production as a function of time, confluent HepAD38 cells were maintained in medium without tetracycline for 11 days. One hundred microliters of the conditioned medium was sampled 1, 4, 7, and 11 days postinduction and analyzed for HBV DNA. From the HepAD38 cell line, HBV DNA could be detected as early as 4 days after induction and increased almost 10-fold between days 4 and 11 (Fig. 3A). In contrast, the levels of viral DNA secreted by confluent 2.2.15 cells changed only about threefold during the same time period. It should be noted that accumulation of viral DNA in 2.2.15 cells strongly depends on the growth phase of the cells and is maximal in confluent cells (29). However, this does not seem to be the case as accumulation of viral DNA in HepAD38 cells occurred in cycling, as well as quiescent, cells (data not shown).

To show that the HBV DNA detected by the dot blot system was rc virion DNA, we precipitated a portion of the medium from day 7 with PEG and analyzed it by Southern blot analysis. As expected, the medium from HepAD38 cells maintained in tetracycline did not contain HBV DNA, whereas the media from both 2.2.15 cells and HepAD38 cells grown in the absence of tetracycline did (Fig. 3B). The difference in the concentration of DNA was approximately 11-fold. In addition, when the PEG-precipitated material from the medium of HepAD38 cells was treated with DNase and RNase but not proteinase K and phenol prior to electrophoresis, the HBV rc DNA comigrated with protein that reacted with anti-core antibody, demonstrating that the rc DNA was associated with HBV core antigen (Ag) aggregates (data not shown).

HepAD38 assay for inhibitors of HBV replication. The ability to tightly regulate production of high levels of HBV prompted us to design a 96-well assay, which we validated by testing several known inhibitors of HBV replication. The compounds we tested were 2′-CDG, 3TC, FTC, ddC and alpha interferon. The EC_{50}s calculated for 2′-CDG, 3TC, FTC, and ddC from the HepAD38 assay were in agreement with those reported in the 2.2.15 assay (Table 1). Multiple tests with FTC and 3TC had shown that these compounds gave highly reproducible results in the HepAD38 assay; therefore, all subsequent test plates contained these two compounds as controls. An example of the autoradiographic data from an assay of ddC and 2′-CDG with FTC and 3TC as controls is shown in Fig. 4. Alpha interferon had no effect on HBV replication in HepAD38 cells. This does not agree with the data reported by Davis and Jansen for the 2.2.15 cell line and may reflect a difference in the types of interferon tested (5).

To ascertain the cytotoxic effects, if any, of the compounds being tested for anti-HBV activity, we determined cell viability after the cells had been exposed to the compounds for 4 days (Table 1). None of the above nucleoside analogs or alpha interferon had a cytotoxic effect at the levels tested. Previous tests with actinomycin D and cycloheximide established that the assay can detect cytotoxic and antiproliferation effects (data not shown).

DISCUSSION

Improvements in the existing in vitro, cell-based systems that model HBV infection should be useful in the study of HBV replication, as well as allow the discovery of new classes of compounds with anti-HBV activity. We have created a stably transfected cell line that contains a cDNA copy of the HBV pg RNA under the control of a tetracycline-responsive promoter. These cells support the replication of HBV under the control of tetracycline. Cells maintained in tetracycline-free medium produce the expected mRNA species and DNA replicative intermediates and release HBV DNA associated with core Ag aggregates into the extracellular environment. Since an immor-

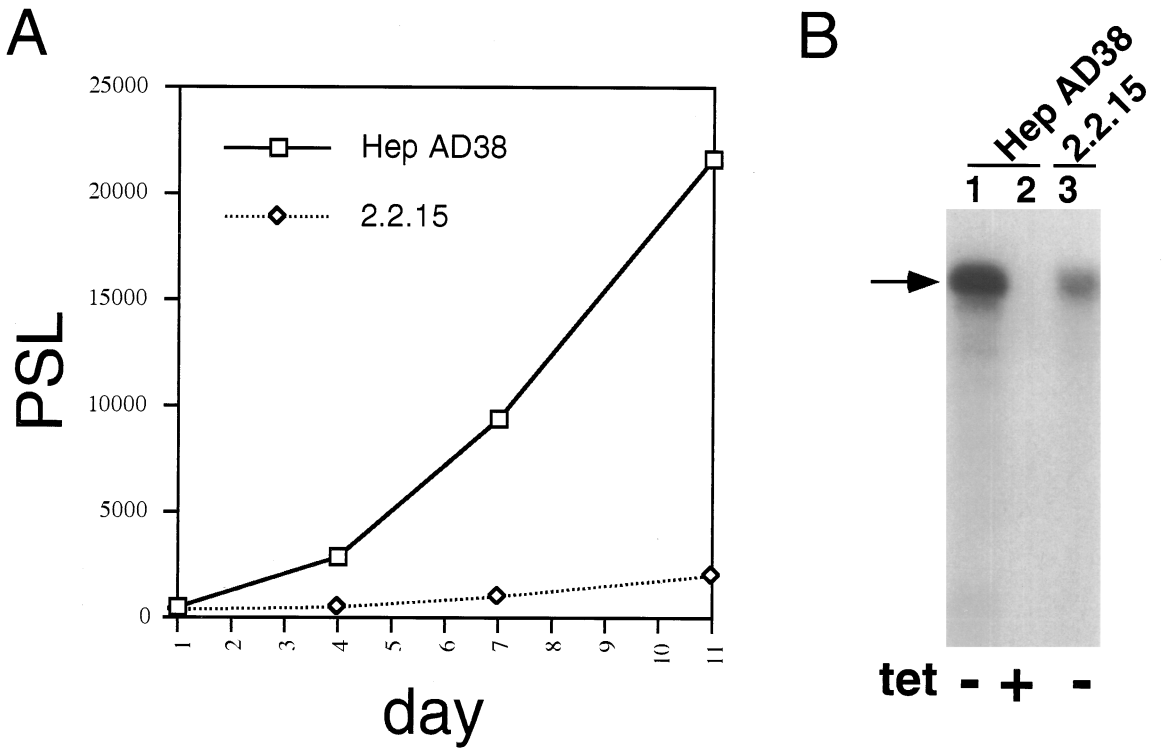

FIG. 3. Production of HBV DNA by HepAD38 and 2.2.15 cells. (A) Cells were plated in 10-mm-diameter wells in the presence of 0.3 μg of tetracycline per ml. Three days later, the cells were washed and medium, either with or without tetracycline, was added. Medium was changed on days 3, 6, and 10 and collected on days 1, 4, 7, and 11 postinduction. The amount of HBV DNA in the medium was determined by quantitative dot blot analysis and quantitated with a PhosphorImager. These data were obtained from a single experiment. Average values for all of the experiments were not used, since there was considerable variation in the efficiency of labeling of the HBV-specific DNA probe between experiments. PSL, arbitrary PhosphorImager units. (B) Virus present in samples from HepAD38 cells (lanes 1 and 2) and 2.2.15 cells (lane 3) from day 7 were precipitated with PEG, and viral DNA was subjected to Southern blot analysis. The arrow indicates rc virion DNA. tet, tetracycline.

talized cell line which can be infected with HBV does not exist, we do not know if these virus core particles are infectious.

We have also performed direct comparisons of intracellular DNA forms expressed by the two cell lines and found no qualitative differences in the DNA species produced (results not shown). However, 7 days after the cells become confluent and are grown in the absence of tetracycline, HepAD38 cells produce approximately 11-fold more viral DNA than do 2.2.15 cells. This increased level of HBV DNA production in the HepAD38 cell line may be due in part to the greater strength of the tetracycline-responsive CMV-IE promoter used in the HepAD38 cell line than the retroviral long terminal repeat promoter that was used to direct the transcription of HBV pg RNA in the 2.2.15 cell line. It also may be due to the disruption of the precore Ag gene in our HBV genomic construct, which occurred when the cDNA was created from the HBV pg RNA, since it has recently been reported that ablation of the HBV precore Ag gene results in a phenotype of increased virus production (23). Moreover, the level of pg RNA produced may be influenced by an advantageous insertion of the transfected DNA into the cellular genome that increases the efficiency of transcription. Lastly, we cannot exclude the possibility that our particular strain of 2.2.15 cells supports HBV replication inefficiently.

The high level of HBV production in the HepAD38 cell line, together with the ability to strictly regulate the commencement of viral replication, will be useful for both academic and industrial applications. For example, in HepAD38 cells, HBV replication does not take place until the cells are induced; therefore, they may be used to study cellular genes that play a role in the HBV life cycle. Cellular mRNAs from induced and uninduced cells can be isolated and compared to determine which cellular RNA species are induced or repressed as a consequence of HBV replication. In addition, this cell line may be useful for determining the kinetics of production and the half-life of the individual replicative intermediates.

The level of HBV produced in induced HepAD38 cells was high enough to format a 96-well microtiter plate-based, high-throughput screen for inhibitors of HBV replication. The use of 96-well microtiter plates is essential for the creation of a high-throughput assay, since this allows many compounds to be screened per assay plate. In addition, this format requires less

TABLE 1. Anti-HBV activity of known HBV inhibitors in the HepAD38 cell line

Inhibitor	HepAD38 cells		2.2.15 cells		Reference
	EC_{50}[a]	TC_{50}[a]	EC_{50}	TC_{50}	
ddC	5.9 ± 1.1	>100	2.8	28[c]	19
3TC	0.03 ± 0.004	>1	0.05	1,722	17
FTC	0.04 ± 0.006	>1	0.04	746	24
2′-CDG	0.02 ± 0.007	>1	0.03	NR[d]	22
Alpha interferon	>1,000[b]	>1,000	280–550	NR	5

[a] Micromolar nucleoside concentrations are shown.
[b] Interferon concentrations are expressed in international units per milliliter.
[c] The TC_{50} for ddC was determined in CEM cells and not 2.2.15 cells.
[d] NR, not reported.

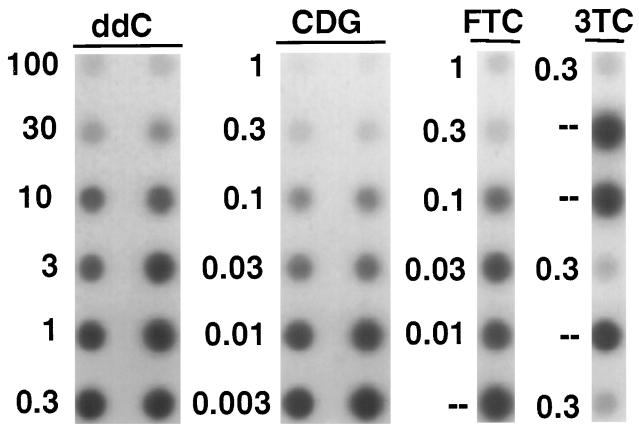

FIG. 4. HepAD38 assay of 2′-CDG and ddC. HepAD38 cells were seeded in 96-well plates in the presence of tetracycline. Three days later, the medium was replaced with medium containing inhibitor but without tetracycline (negative control wells contained medium without inhibitor or tetracycline). Micromolar inhibitor concentrations are shown. FTC and 3TC were used as controls for the inhibition of HBV replication. Ninety microliters of medium from each well was assayed for the presence of HBV DNA by dot blot analysis as described in Materials and Methods. The data were obtained from a single nylon membrane; however, the image was separated into four columns for ease of labeling. – –, no-drug control (i.e., cells were induced but not treated with inhibitor).

compound and tissue culture medium, thus reducing the cost of the assay. The 96-well format also would allow the incorporation of multichannel pipettors, automatic pipetting stations, and robotics into the assay procedure, thereby reducing the hands-on time of screening while increasing the consistency of the assay. In addition, the assay can be completed in approximately half the time necessary for the traditional 2.2.15 assay. Moreover, since commencement of HBV replication is inducible in the HepAD38 cell line, with no intracellular HBV replicative intermediates present prior to induction, the long washout period required with 2.2.15 cells is eliminated. We have validated the assay by screening several known inhibitors of HBV replication and showing that the EC_{50}s of these compounds were equivalent in the two assay systems. To date, the only disparity found was in the antiviral activity of alpha interferon, which was probably due to the types of alpha interferon tested rather than to differences in the assays.

The above advantages make it economically feasible to use the HepAD38 assay for large-scale screening of diverse chemical libraries in order to identify new classes of inhibitors of HBV replication. By using the procedure described above, one person is able to complete two assays (i.e., 10 plates per assay; five compounds assayed in quadruplicate at two concentrations per plate) in a 7-day period, resulting in the screening of approximately 100 compounds per week for anti-HBV activity and cytotoxic effect. This results in one person having the ability to screen an estimated 5,200 compounds per year. However, with the inclusion of robotic stations to perform the dilution of compounds and the distribution of medium into 96-well plates, this number should be increased to approximately 16,000 compounds per year. This would be at least a 15-fold improvement over the existing 2.2.15 assay if compounds were screened at two concentrations in quadruplicate.

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